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MUCILAGE IN YELLOW MUSTARD (*BRASSICA HIRTA*) SEEDS

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Abstract

Release of mucilage from yellow mustard (*Brassica hirta*, also known as *Sinapis alba*) seed coats (hulls) was studied by optical and scanning electron microscopy. Micrographs were obtained of the mucilage which had exuded from briefly moistened seeds and dried subsequently in the form of small droplets on the seed surface.

The mucilage collected from the seed surface and mucilage isolated on a larger scale from seed hulls was hydrolyzed with sulfuric acid and the hydrolyzates were analyzed for sugar composition. Galactose, glucose, and galacturonic acid were found to be major components and mannose, arabinose, xylose, and rhamnose were minor components. Individual neutral monosaccharides were identified by paper chromatography, and paper electrophoresis, and finally quantitated by gas-liquid chromatography and characterized by combined gas-liquid chromatography chemical-ionization mass-spectrometry of the derived alditol acetates. Mucilage from both sources was found to be identical.

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Introduction

Seeds of the genus *Brassica* are known to contain varying amounts of mucilage. The mucilage is of particular importance in mustard seeds because it contributes to the consistency of prepared mustard (Weber et al., 1974). Of several mustard varieties, yellow mustard (*B. hirta*) is particularly rich in mucilage and contains approximately 2% of it (Bailey and Norris, 1932; Woods and Downy, 1980), and for that reason, the seed is an important commercial commodity. A similar mucilage is found in rapeseed (Van Caeseele et al.; 1981, Yiu et al., 1982; Van Caeseele and Mills, 1983).

In some stored mustard seeds, a small amount of a whitish substance was found on the seed surface. Seeds, which had been moistened and immediately dried, were found to be glued together by a similar substance, showing thereby that the mucilage in the seeds rapidly exuded following exposure of the seeds to moisture. The release of mucilage from seeds immersed in water was used as an indicator of the presence of the mucilage in rapeseed cultivars (Van Caeseele and Mills, 1983).

Previous cytological studies, which were carried out using light microscopy of seed coats (hulls) in an aqueous medium and using electron microscopy of hulls embedded in a resin revealed that in rapeseed the mucilage developed between the plasmalemma and the outer tangential wall of the epidermal cells and that at maturity, the seed epidermal cells were totally devoid of cytoplasm and engorged with mucilage (Van Caeseele et al., 1981).

The present study was designed to examine the development of the efflorescence on the seeds by optical microscopy and scanning electron microscopy and to compare its composition to the mucilage isolated from the seed hulls. The polysaccharide components of the mucilage in mustard seed have been known and contain an arabinan (Hirst et al., 1965; Rees and Richardson, 1966; Aspinall and Cottrell, 1971), a xyloglucan (Gould et al., 1971) (amyloid), and pectic materials (Rees and Wight, 1969). Results of analyses such as chemical ionization and mass spectrometry are reported in this paper.

Materials and Methods

Seeds. Seeds of yellow mustard (*B. hirta*) were obtained from the 1980 harvest from Outlook Isolation (Ref. 80-7500040-01). The seeds were examined intact; a part of the sample was gently ground in a Krupp 75 coffee mill so as to break the hulls away from the seeds and retain the integrity of the hulls at the same time.

Extraction of mucilage from mustard hulls. Yellow mustard hulls (Batch FRI-71-29), were extracted with boiling water (1:16, w/v) for 35 min and centrifuged at 2000 rpm for 20 min, yielding a viscous aqueous solution (Weber et al., 1974). Isopropanol was added to the filtrate to a final concentration of 70% (v/v) and the resulting precipitate (mucilage A) was separated using an organdie cloth, washed with 70% isopropanol, air-dried, and pulverized.

Isolation of mucilage from hulls of intact mustard seeds. The yellow mustard seeds were moistened with water, excess moisture was allowed to evaporate, and the sticky material on the outside of the hulls was transferred by gently rubbing the seeds on the surface of a glass plate. The dried material was isolated by scraping the glass surface, dissolved in water, and recovered by precipitation in 70% isopropanol (mucilage B).

Chemical analyses. Gas-liquid chromatography (GLC) was performed with a Varian Vista 6000 Gas Chromatograph, with flame-ionization detectors, glass column (1524 x 3.18 mm i.e. 5 ft x 0.125 in) packed with 3% OV.225 on Chromosorb WHP (80-100 mesh), a temperature program 100 → 230°C at a rate of 2.5°C/min and a nitrogen flow rate of 30 mL/min. Peak areas were evaluated with a Pye-Unicam CPD1 Computing Integrator. Combined gas-liquid chromatography chemical-ionization mass-spectrometry (GLC-CI-MS) (Horton et al., 1974) was performed with a Finnigan Incos MAT-312 system with butane as the reagent gas, operating with CI ion Pouree temperature 160°C, 250 eV, filament emission 0-5 mA, accelerating voltage 3 kV, electron multiplier 2 kV, scanning range 60-500 at 2 s/scan, and a column (1829 x 6.35 mm i.e. 6 ft x 0.25 in) of OV-225 on Chromosorb WHP (80-100 mesh), a temperature program 120 → 230°C at a rate of 5°C/min and a helium flow rate of 30 mL/min. Optical rotations were measured with a Perkin-Elmer 141 polarimeter.

Descending paper chromatography was performed on Whatman No. 1 paper using organic phases of an ethyl acetate-pyridine-water (8:2:1) or a 1-butanol-acetic acid-water (4:1:5) system.

Paper electrophoresis (Haug and Larsen, 1961) was performed on Whatman No. 3 MM paper in a borate-calcium chloride buffer (pH 9.2) at a potential gradient of 14 V/cm. Sugars were detected with aniline hydrogen phthalate. Concentrations were carried out at 35°C in a rotary evaporator.

Hydrolysis of the mucilage samples (2-3 mg) was performed with 1 M sulfuric acid (0.3 mL) for 3 h at 100°C or with aqueous 72% sulfuric acid (0.12 mL) at 5°C for 3 h followed by dilution to 1 M acid and heating for 3 h at 100°C. The hydrolyzates were neutralized with barium carbonate. The sugar samples (3 mg or less) were reduced with sodium borohydride, and the alditol acetates were acetylated essentially as described by Bjorndal et al., (1967) except that the acetylation was carried out at 100°C for 1 h followed by storage overnight at 22°C. Portions (2-3 mg) of the hydrolyzed sugars were fractionated on columns (70 x 5 mm) of Dowex 1x2 (CO_3^{2-}). Elution with water (5 mL) yielded neutral fractions which were concentrated, reduced with sodium borohydride, acetylated, and examined by GLC and GLC-CI-MS.

Light microscopy. Mature yellow mustard seeds were fixed in 5% glutaraldehyde in 0.01 M potassium phosphate buffer, pH 7.2 at 4°C for 48 h. After fixation, the seeds were mounted on cold object discs and frozen in Histo Prep medium (Fisher Scientific Co., Fair Lawn, N. J.) at -20°C. Frozen sections, 6-8 µm thick, were cut from the seeds using a Reichert-Jung Cryo-cut E microtome. Alternatively, the fixed seeds were embedded in glycol methacrylate (GMA) resin as described by Yiu et al. (1982). They were dehydrated in an alcohol series in the order of methyl cellosolve, ethanol, *n*-propanol, and *n*-butanol. The dehydrated seeds were then infiltrated with GMA monomer for 3-5 days prior to polymerization at 60°C in gelatin capsules. Sections 2 µm thick were cut using glass knives in a Sorvall Porter-Blum microtome, affixed to glass slides, and stained with one of the following dyes:

Toluidine Blue O, 0.05% (w/v) in 0.1 M potassium phosphate buffer, pH 6.5, for 1-2 min. After staining, the sections were briefly rinsed in distilled water, air-dried, mounted in immersion oil, and examined using brightfield illumination.

Calcofluor White, 0.01% (w/v) aqueous solution, for 1-2 min. The sections were rinsed in water, air-dried, mounted in non-fluorescent immersion oil, and examined for fluorescence using filter system FC I (see below).

Congo Red, 0.01% (w/v) aqueous solution, for 1-2 min. The sections were briefly rinsed in water, dried, mounted in non-fluorescent immersion oil, and examined for fluorescence using filter system FC III (see below).

Fluoresceinated wheat germ agglutinin. The sections were incubated with 1.2 mg/mL of fluorescein-labelled wheat germ agglutinin (Cedarlane Lab. Ltd., Hornby, Ont., Canada) in 0.01 M sodium phosphate buffer, pH 7, at 22°C for 1-2 min. Stained sections were rinsed thoroughly with distilled water, air-dried, mounted in oil, and examined under the microscope using filter system FC II (see below).

The sections were examined using a Zeiss Universal Research Photomicroscope equipped with both a conventional brightfield illuminating system and a III RS epi-illuminating condenser combined with an HBO 100 W mercury-arc illuminator for fluorescence analysis. Three fluorescence filter combinations, each with a dichromatic beam splitter and an exciter/barrier filter set for maximum transmission at 365 nm/ >418 nm (FC I), 450-490 nm/>520 nm (FC II), and 546 nm/>590 nm (FC III), were used for fluorescence examination. Photomicrographs were taken on Kodak Tri-X pan film.

Scanning electron microscopy (SEM). Dry yellow mustard seeds were examined intact. They were also cut in half and the hulls were separated and mounted for examination of their outer and inner surfaces. In parallel experiments, seed hulls were extracted with 3 changes of boiling water for the total time of 3 h. The extracted hulls were dried in air and prepared for SEM.

Release of mucilage from the seeds was studied in seeds which had been spread in a single layer on filter paper moistened with distilled water in a Petri dish; the seeds were dried over concentrated sulfuric acid following exposure to moisture for 1, 2, 3, and 4 h. The seeds and separated seed hulls were prepared for SEM by mounting on aluminum stubs using silver cement and coating with gold (approx. 20 nm) by vacuum evaporation. The seeds and hulls were examined in a Cambridge Stereoscan Mark II scanning electron microscope operated at 20 kV.

Results and Discussion

One of the simplest methods of detecting the presence of mucilage in mustard seeds is by wetting the seeds with water and examining them under a stereomicroscope several minutes later. The appearance of a gelatinous halo surrounding the wetted seed indicates the presence of mucilage on the seed surface. More elaborate microscopic techniques were required in order to examine

the structure of mucilage in detail. Several microscopic methods were used for the detection of yellow mustard mucilage in this study.

Toluidine Blue O, a metachromatic dye which was found to be useful for staining various plant structures (O'Brien et al., 1964, and Yiu et al., 1983) including mucilage in rapeseed, *B. campestris* cv. Candle (Van Caeseele et al., 1981), was used for staining GMA sections of the yellow mustard seeds. Microscopic examination under brightfield illumination revealed a swollen epidermis, the outer seed coat layer of the seed, with visible pink striated contents and thickened cell walls (Fig. 1) indicating the presence of mucilage. Fluorescence microscopy was used to achieve a higher resolution of the structures. Several fluorescent dyes or reagents (Calcofluor White, Congo Red, and fluoresceinated wheat germ agglutinin) that have known affinities for specific polysaccharides (Yiu et al., 1982, Miller et al., 1984) were employed in this study. While all three reagents were useful in detecting the presence of mucilage in the frozen sections of the mustard seeds, Calcofluor White was the only effective fluorescent marker for the GMA sections. One such section stained with Calcofluor White is demonstrated in Fig. 2. It shows the structural relationship between mucilage and the rest of the seed coat layers. The structure of the mustard seed has already been described elsewhere (Winton and Winton, 1932; Vaughan et al., 1976) and will not be repeated here. Briefly, the mustard seed coat (hull) consisted of an epidermal layer where mucilage was detected, a sub-epidermis, a palisade layer, and a pigment layer. The aleurone layer of the endosperm remained associated with the seed coat during dehulling. Although Congo Red had been shown to stain mucilage well in GMA sections of both rapeseed (Yiu et al., 1982) and oriental mustard seeds (Holley et al., 1983), it failed to stain the GMA sections of yellow mustard seeds. It is not certain whether this finding reflects any chemical or dye-affinity differences between the mucilage of yellow mustard and mucilage of rapeseed and oriental mustard. More studies are required in order to understand the mechanism of interaction between Congo Red and different varieties of mucilage. On the other hand, it could be speculated that owing to the relatively large molecular size of fluoresceinated wheat germ agglutinin, the lectin was not able to penetrate the GMA resin easily and, consequently, did not stain the mucilage as well as it did in the frozen sections. The findings were similar to results obtained with rapeseed sections which were used as controls. Although the use of fluoresceinated wheat germ agglutinin was thus limited to frozen sections, which did not reveal structural details because of their thickness, the lectin served as a specific probe staining no other seed coat structures but the mucilage (Fig. 3). Besides, informative results can be obtained from frozen sections within a relatively short time (1-2 days) as compared with GMA sections which require more than a week to complete the sample preparation procedures. In spite of being relatively rapid and simple to perform, the techniques of light microscopy do have some disadvantages. Aqueous fixation is usually required for sample preparation. Fixation using glutaraldehyde vapour is possible but is not as effective as glutaraldehyde solution. In addition, most of the staining methods use aqueous solutions. The presence of water may lead to the emergence of mucilage at the seed surface that does not occur in dry seeds and this can be regarded as an artefact.

Scanning electron microscopy (SEM) was used to

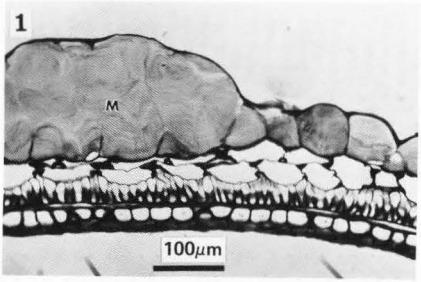

Fig. 1. A Toluidine Blue O-stained section of a yellow mustard seed coat embedded in glycol methacrylate showing the striated structure of mucilage (M).

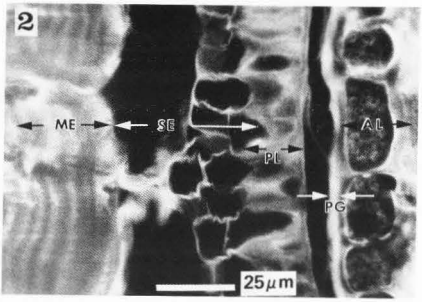

Fig. 2. A Calcofluor White-stained section of yellow mustard seed embedded in glycol methacrylate showing the structures of the seed coat, which consist of a mucilaginous epidermis (ME), a sub-epidermis (SE), a palisade layer (PL), a pigment layer (PG), and an aleurone layer (AL).

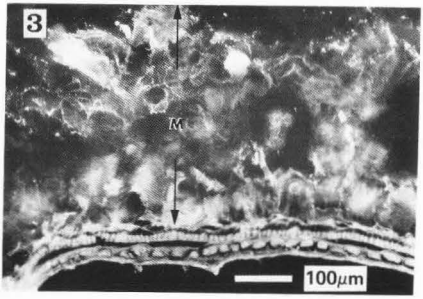

Fig. 3. A frozen section stained with fluoresceinated wheat germ agglutinin showing the presence of mucilage (M) at the seed coat surface.

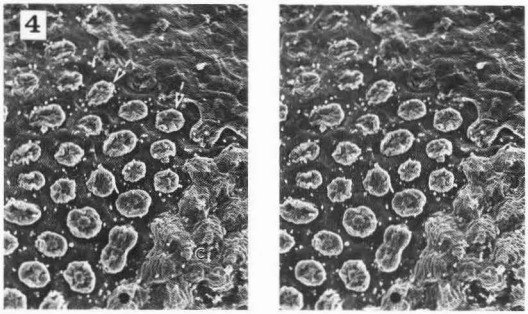

Fig. 4. Mucilage emerged from the hull of a seed moistened for 1 h and subsequently dried.
The mucilage is in the form of individual minute droplets (arrows) or in the form of droplets coalesced into a stratified cover (C). In this stereo pair of micrographs (12° angular separation), two dots have been provided to facilitate focussing of the eyes.

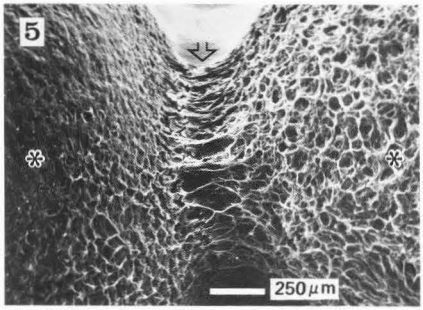

Fig. 5. Mucilage (arrow) cementing two yellow mustard seeds (asterisks) following their exposure to moisture for 4 h and drying.

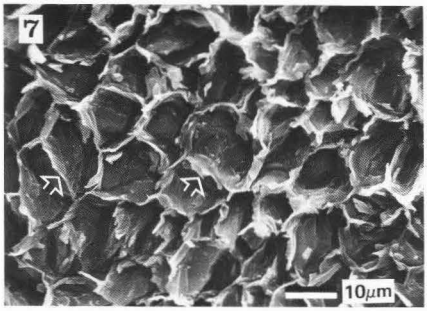

Fig. 7. Mechanical removal of the inner lining from the internal surface of a seed hull reveals broken cells (arrows).

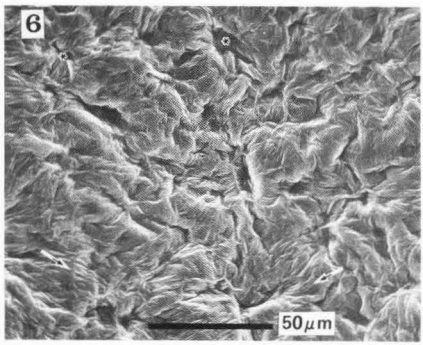

Fig. 6. Internal surface of an intact seed hull. Regular depressions are marked with asterisks. Arrows point to fine wrinkling of the surface.

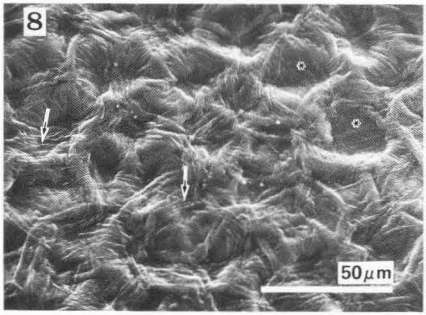

Fig. 8. Internal surface of a seed hull extracted with boiling water for 3 h and subsequently air-dried. Depressions (asterisks) in the surface and fine wrinkling (arrows) are similar to those in an intact hull.

record the exudation of mucilage from a seed exposed to moisture for 1 h and the result is shown in a stereo pair of micrographs (Fig. 4). This treatment altered the seed surface compared to the images of untreated seeds shown by Mulligan and Bailey (1976). In some places on the seed surface, small droplets of the mucilage had already started to coalesce and form a stratified mucilage cover; coalescence of the mucilage droplets was common to seeds exposed to moisture for periods longer than 1 h. The emergence of the mucilage from isolated hulls was also observed and resembled the emergence of mucilage from rapeseed as documented by Van Caeseele and Mills (1983). A compact layer of mucilage dried on the seed surface (4 h exposure to moisture) is shown in Fig. 5. It was this layer of mucilage, which was the subject of the present study. The mucilage was initially isolated by gently scraping the dried seeds but later a more efficient technique was developed whereby wet seeds were rolled on a glass plate and the mucilage thus transferred from the seeds to the glass plate was dried and scraped off for analysis.

The inner surface of the hulls was also examined by SEM. The surface of an intact hull is shown in Fig. 6. There are depressions in the surface which appears as if coated with a finely wrinkled film. This film can be observed by a naked eye as a whitish material and can be easily removed with a pair of tweezers. Its mechanical removal exposed the underlying cells (Fig. 7). Exposure of the hull to boiling water for 3 h and consecutive air-drying did not alter this surface to any considerable extent (Fig. 8) except that the depressions are somewhat deeper. The emergence of the mucilage from hulls immersed in water was observed only on the outer hull surface and was not observed on the inner surface.

Results of chemical analyses following hydrolysis of the mucilage with 1 M as well as 72% sulfuric acid, paper chromatography, and paper electrophoresis are presented in Table 1. The results indicate that the materials were free from low molecular weight sugars and were essentially free from protein contaminants. The analytical data, $[\alpha]_D$ values, and the hydrolysis (GLC-CI-MS of the derived alditol acetates) confirmed the identity of the component sugars and indicated the molar proportions of the parent neutral sugars, constituting samples A and B, to be very close. A typical gas chromatogram of the alditol acetates from mucilages A and B is shown in Fig. 9 and data are summarized in Table 2. This evidence demonstrates that the two samples were identical materials except that mucilage B had significantly lower pectin and protein contents (Table 1).

The hydrolysis data further revealed certain aspects of the polysaccharide composition of the mucilage. The presence of uronic acids, and rhamnose residue, and possibly an aldobiuronic acid, probably the commonly occurring (1→2)-linked (galactosyluronic acid)-rhamnose, showed that a major portion of the mucilage is composed of polysaccharides of the pectic type. The presence of glucose, galactose, and xylose residues and the different galactose and glucose ratios (1:0.23, M H_2SO_4; 1:0.62, 72% H_2SO_4, mucilage A) and (1:0.31, M H_2SO_4; 1:0.72, 72% H_2SO_4, mucilage B) were indicative of the presence of a xyloglucan (amyloid). These differences originate from different degrees of hydrolysis of the cellulose backbone in amyloids. Both polysaccharides have been reported in mustard (Gould et al., 1971; Rees and Wight, 1969).

The present results could not demonstrate unequivocally the presence of an arabinan component but the presence of arabinose residues in conjunction with literature reports (Aspinall and Cottrell, 1971, Hirst et al., 1965, Rees and Richardson, 1966) strongly suggest that the arabinan was also present.

Table 1
ANALYSIS OF YELLOW MUSTARD MUCILAGE

Fraction	A	B
Optical rotation $[\alpha]_D$ (0.1 M NaOH)	0±5°	0±5°
Hydrolysis results:*		
Galactose	major	major
Glucose	major	major
Mannose	minor	minor
Arabinose	minor	minor
Xylose	minor	minor
Rhamnose	minor	minor
Galacturonic acid	+	+
Aldobiuronic acid	+	+
Analytical data (%):		
N	0.97	0.22
Ash	6.85	5.96
Moisture	7.13	8.80
Acetyl	0.00	0.00
Methoxyl	2.24	1.88
Uronic acid	30.50	18.19

* *Obtained by paper chromatography and paper electrophoresis.*

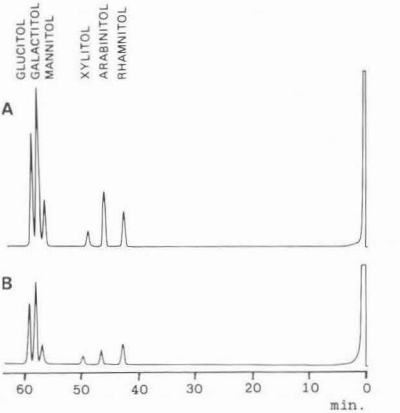

Fig. 9. Gas-liquid chromatograms of neutral sugars, in the form of their alditol acetates, as obtained after hydrolysis with 72% H_2SO_4. Relative retention times are listed in Table 2. A = yellow mustard hull mucilage; B = yellow mustard mucilage scraped from the seed surface.

Table 2
ANALYTICAL AND COMBINED GAS-LIQUID CHROMATOGRAPHY CHEMICAL IONIZATION MASS SPECTROMETRY (GLC-CI-MS) DATA FOR SUGARS FOLLOWING HYDROLYSIS (72% H_2SO_4), REDUCTION, AND ACETYLATION

Acetate of	Retention time relative to galactitol	Molar ratio		CI-MS data: m/z [relative abundance, (%)]
		A	B	
Glucitol	1.02	0.62	0.72	$[MH]^+$ 375 (100), 257 (15), 376 (15)
Galactitol	1.00	1.00	1.00	$[MH]^+$ 375 (100), 257 (15), 376 (15)
Mannitol	0.98	0.29	0.27	$[MH]^+$ 375 (100), 257 (15), 376 (15)
Xylitol	0.85	0.08	0.09	$[MH]^+$ 303 (100), 185 (15), 304 (15)
Arabinitol	0.80	0.29	0.27	$[MH]^+$ 303 (100), 185 (15), 304 (15)
Rhamnitol	0.74	0.20	0.26	$[MH]^+$ 317 (100), 199 (15), 318 (15)

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